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Biotic Contamination and Possible Ways of Sterilization: A Review with Reference to Bamboo Micropropagation.

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ABSTRACT

Multipurpose use of bamboo in rural life makes it as poor man's timber in Asian countries. Deforestation and industrialization leads to destruction of natural forest. To replenish, a rapid plantation of bamboo could be one of the possible solutions. Bamboo is propagated mainly by vegetative methods though it is not suitable for large scale plantation because of several limitations. Micropropagation is gaining importance for large scale propagation because of its capability in raising huge number of true to type propagules in a limited space in very short span of time. Like any other plant, the chief constraint of bamboo micropropagtion is in vitro contamination arises from several sources including explants. Most of the contaminants are reduced by maintaining aseptic conditions. The surface adhering microbial contaminant (Epiphytic) is usually checked by using several available surface sterilants. But the endophytic contaminant (present within the explants) is not easily controlled. Endophytic fungus could be controlled by using systemic fungicides but controlling bacteria is again more troublesome. Antibiotic with broad spectrum activity coupled with low phytotoxicity is prerequisite to get better results. Treatment duration and type of antibiotic are the critical factor to reduce the contamination. But unscientific use of antibiotic may lead to the development of resistant microbial strains. That is why antibiotic selection after identification of the contaminants may be an efficient way to counter this problem. The present review is done on use of antibiotic controlling bacterial contamination during micropropagation special reference to bamboo.

Key words: Biotic contamination, sterilization, bamboo, micropropagation

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INTRODUCTION

Bamboo is considered as the fastest growing perennial, evergreen plant belonging to subfamily Bambusoideae under the family Poaceae [1, 2]. Multipurpose utility of bamboo made it 'My brother' in Vietnamese, in Chinese it is called as 'Friend of the people' and in India it is known as 'Green gold" or 'Poor man's timber'[3]. In spite of several economic importance, the productivity is less in India than china and most of bamboo stock are available from natural forest [4]. Reduction of natural forest is increasing day by day due to uncontrolled deforestation and human activities such as industrialization [5]. So, the multiplication and conservation of the bamboo species in its natural environment is urgently needed [6]. Bamboo is propagated through either of seed or vegetative methods [7] though both methods have several disadvantages. Seed based propagation is limited due to long and unpredictable flowering cycle, poor seed setting, short seed dormancy period, high seed sterility, low seed viability, high seed-borne infections [8]. Vegetative propagation method has also several disadvantages such as bulkiness of cutting materials [9, 10, 11], seasonal dependency [12] and low multiplication rate [13]. Plant tissue culture is already proved as a reliable tool to produce large number of genetically identical plant *in vitro* condition [14]. So bamboo propagation by plant tissue culture method and its plantation in natural environment are essentially becoming an alternative way to restore the forest in natural environment. Like any technique, the micropropagation has also limitations besides its several advantages. Though juvenile plants are more suitable than matured plant for micropropagation [15] but both of adult and juvenile plants are suitable for tissue culture of bamboo [11,16]. The adult explants have several problems like endogenous contamination [16], low growth rate and rooting [17, 18, 19]. Contamination, especially biotic i.e. fungal and bacterial contaminations, is considered as single most important damaging factor of *in vitro* culture of plants [20,21,22]. The present review was aimed to address those contaminants and its possible ways of eradication with special reference to micro-propagation of bamboo.

BIOTIC CONTAMINATION IN PLANT TISSUE CULTURE

Microbial contaminants may arises from different sources like infected plant materials, improper tissue culture technique and poor laboratory condition [23, 24, 25, 26, 27]. Explant contamination is related to several factors like source of explants and environment [21]. Fungal and bacterial contamination is one of the serious problems for the micro-propagation of woody plant species [28, 29, 30]. Among fungal contamination, presence of systematic fungal contamination is the most problematic issue of micropropagation of mature woody species [31].The fungal contamination was considered as most predominant factor in *B. balcooa* during tissue culture [29]. Fungal contamination may also arises from explants itself or air or during culture [31, 32], also associated to the indoor air, tables/walls, and human skin [33]. On the contrary, the latent contamination of bacteria is the most problematic issue for *in vitro* propagation of several species of *Bambusa* [30]. In plant like bamboo, the nodal explants have large intercellular spaces, which are exposed during cutting before surface sterilization and promote the entry of bacterial and fungal spores into explant. As a result they are unable to control by the surface sterilization protocol and further found as contamination advance stages in medium [34]. These microorganisms are competing adversely with plant for their growth since the media considered as good nutrient source for them [20]. Presence of this microorganisms leads to increase of plant mortaltity, create variation in growth (reduction of shooting proliferation and rooting), tissue necrosis even plant death [35].

EPIPHYTIC VS ENDOPHYTIC

The contaminant in culture media may have immediate or latent expression in which it remains dormant for long time [36]. Epiphytic bacteria live on plant surfaces [37] and can be removed through chemical disinfectants. In contrast, the endophytic microbes i.e., microbes that colonize living, internal tissues of plants, without causing any immediate negative effect [38,39] and are not easily eliminated by simple surface-sterilization methods [36,40,41]. For this reason the existing contaminants are generally controlled by using either antibiotic or fungicide [42, 43, 44]. That is why the antibiotic therapy is getting much importance to solve this problem [45]. Now a day, there are several alternative ways to control the contaminants reported by several authors such as through light and heat [46], micro wave and hot water treatment [47]. But till now most researchers depends on chemicals to control the contaminants during *in vitro* propagation of plant.

SELECTION OF ANTIBIOTIC

Elimination of the latent contamination in culture may be done by using several antibiotics. But type, application and treatment duration of antibiotics vary plant to plant [48]. In general antibiotics are broadly classified into two groups i.e; bactericidal (kills bacteria) and bacteriostatic (prevent bacterial growth). Several researchers experimentally proved that the uses of bactericidal are more advantageous over bacteriostatic antibiotic to control the endophytic contamination if there is no side effect of antibiotics in explants [49, 50, 51]. An ideal antibiotic in such case should be attributed as stable, easily soluble in nature, not affected by components and pH of the medium, lacks side effects, broadly active, non-resistance inducing, inexpensive and non-toxic to humans [52,53]. But tremendous use of antibiotics is not recommended because of its phytotoxicity and selection for resistance strains [44]. Since most of antibiotics have narrow target range for bacteria, combination of antibiotics were found better to reduce contamination as well as damaging plants due to their synergistic effect [54]. But antibiotic having broad range of target may shown better response than the combination of antibiotics [55]. Similar to antibiotic, idle fungicide should be nontoxic to plant cell and broad spectrum fungicidal activity [56]. Since continuous use of single antibiotic often leads to antibiotic resistant microbial contaminants on culture, combination of different antibiotics is better option to counter the problem [41, 51]. This approach if suitable to solve the problem then there is a need of modification in concentration of antibiotics to lowering down the phytotoxicity level of antibiotic on plant since effective concentration for both antibiotic may affect the plant [51]. Combination of antibiotics will kill contaminants without damaging the plant. Use of combination of bactericides in bamboo/woody plants tissue culture, which has limited uses of antibiotics, may be propoted to avoid the unwanted effect of microbial contaminants on the growth of plant.

ANTIBIOTIC IN MODERN ERA

Till now several attempts have been taken to reduce the contamination using the antibiotics. But it has several problems. Removal of bacteriostatic effect of antibiotic leads to continue the growth of bactetia [52, 57, 58, 53]. Besides that rapid use of antibiotic may leads to phytotoxicity [59] and development of resistant microbial strains [60, 61]. Long duration exposure of cells or tissues to antibiotics may also leads to change in genetic makeup of organelles (the cytoplasmic genes or cytoplasmic DNA) as well

as development of resistance in bacterial cells [62]. Therefore characterization of contaminants (type) before the antibiotic therapy is right way to counter the problem since it may leads to appropriate selection of antibiotic. This method is reported by several authors for several plants including; Hazelnut [41], *Withania somnofera, Piper nigram, Piper colubrium* and taxus *Baccata*sub sp., *Wallichiana* [61]; *Jatropha curcus* [63], including bamboo (*Guadua angustifolia* Kunth) [64]. Use of Plant Preservative Mixture™ (PPM) (Plant Cell Technology, Washington, D.C.) is also an alternative option to control the contamination reported by several authors. This chemical is mixture up of methylchloloisothiazolone and methylisothiazolone [44], is a biocide compound, heat stable and effective against a wide spectrum of common *in vitro* contaminants [65]. PPM is effective against both bacteria and fungi and unlike conventional antibiotics, can be autoclaved in the media [66]. These characteristics of PPM make it an attractive alternative to using conventional antibiotics and fungicides in plant tissue culture. Antibiotics as media components are also reported in bamboo (*Guadua angustifolia* Kunt) [67].

EDAPHIC FACTORS VIS-À-VIS CONTAMINATION

It is evident from several literatures that the chance of contamination during *in vitro* culture and seasons for collecting explants has a strong positive correlation. Explant collected at rainy season is having higher bud breaking coupled with high chances of contamination. More than 60% endogenous contamination during rainy season reported in *B. nutans* [68]. Collection of nodal explants (*Arundinaria callosa* Munro) during rainy season showed 30% more contaminations than that of after monsoon season [69]. Quite similar results were reported in *D. asper* [70, 71]; in *D. hamiltonii* [72] and in *D. giganteus* [73]. It could be suggested to avoid collection of explant during rainy season.

SURFACE STERILANTS FOR BAMBOO TISSUE CULTURE

Bamboo micropropagation most widely used surface sterililants are Bavistin and Mercuric chloride. Sodium or Calcium hypochloride and Hydrogen peroxide are also reported by several workers for several bamboo species. Mercuric chloride (0.1%) was found better than Sodium hypochloride (0.05, 0.1 and 0.2%) in *B. tulda* [74] after treatment of 10 minutes. Treatment of 20 minutes under Mercuric chloride (0.2%) was found better than 10-20 minutes treatment of Calcium hypochloride in *D.strictus* [8]. 0.1% Mercuric chloride was better than Sodium hypochloride (2%) and Hydrogen peroxide (10%) in *B. Ventricosa* (treatment durations were not same for this treatments) [75]. Similar report in other plant reported in nodal explants of *Aconitum heterophyllum* [76]. They found 0.1% Mercuric chloride was superior over Hydrogen peroxide (10%) and Sodium hypochloride (1.5%). Although, the treatment with high concentration (0.2%) of Mercuric chloride for 10-15 minutes increased the chance of aseptic culture in *D. strictus* [77], it reduces the bud breaking percentage of plant reported in *B. wamin* [78]. On the contrary, lower duration of 0.1% Mercuric chloride increases high survival rate in *B. ventricosa* [75]. Pretreatment of Bavistin on nodal explants reduces the chance of fungal contamination in *G. atroviolaceae* [79]. Negative impact of Calcium hypochloride as surface sterilant (discolorization of plant) in *Guadua angustifolia* Kunth reported by [67]. 1% Bavistin is reported as most widely used concentration as surface sterilants during *in vitro* propagation of bamboo. Though there are exceptions also, Bavistin reported @ 0.1% in *Bambusa nutans* Wall [68]; 0.5% in *B.nutans* [80]; 0.2% in *D. strictus* [81]. Besides Bavistin, other fungicides that are reported till date as surface sterilants for *in vitro* propagation of bamboo are Benomyl and Mancozeb. Though Bavistin and Benomyl are made up of

same active component benzimidazole but Benomyl is considered as most effective chemical to control fungal contamination [22]. Use of this chemical are very limited in comparsion to Bavistin for *in vitro* propagation of different bamboo species; Benlate (benomyl) @ 1 gm/l reported in *D.giganteus* and *B.vulgaris* [82]; as media component in *Dendrocalamus giganteus* Munro [73], 0.1% in Bambusa vulgaris 'Striata' [83], in *Guadua angustifolia* @ 2g/l [67]. Mancozeb is reported @ 0.1% in *B. balcooa* [84], *B. nutans* @ 0.1% [85]. The duration of surface sterilization that depends on the size and nature of explants has significant role in the success rate of macropropagation. Effect of sterilization is depending on several factors such as sterilization type, concentration and time of treatment with the sterilizing agent [86]. The reduction of contamination may vary from species to species and also depends on the chemicals which are used. During surface sterilization, the cutting ends are entry point of the active compound of the surface sterilant, so profound penetration of the chemicals results toxic effect on explants and slow down the growth of plant [34]. This entire chemical fruitfully eradicates the surface contaminations but has limitation in controlling the endophytic contamination. Now a day, the antibiotics are also being used during surface sterilization.

ANTIBIOTIC AS SURFACE STERILANTS IN BAMBOO

Microbial contamination using antibiotics are reported by several workers during *in vitro* propagation of different plants i.e; Rubber (*Heveabrasiliensis*Muell.Arg) [87], Hazelnut [41], *Withania somnofera, Piper nigram, Piper colubrium* and taxus *Baccata* subsp., *Wallichiana* [61], *Jatropha curcus* [63], Banana [88], Orange tree i.e; *Citrus sinensis* L. Osbeck cv. Madame Vinous and Sweet orange trees i.e; *C. sinensis* cv. Valencia [28]. In bamboo, attempts have been taken to reduce the endophytic contamination during *in vitro* propagation using antibiotics as surface sterilant. Agri-mycin and Benomyl in *Guadua angustifolia* [67], mixture of Gentamycine and Mancozeb [84], mixture of Bavistine and Bacteriomycine [89], Germicide, Teepol and savlon [90] for *B.balcooa*, Streptomycin Sulfate for *B. nutans* [68], *D.*membranaceus Munro [91], Streptomycin and Kanamycin for *Dendrocalamus giganteus* [92] ,Mancozeb and Streptomycin Sulphate in *B.nutans* [85] were reported as surface sterilant during in *vitro* proapagation of these species. Use of Streptomycin, Riffampicin, Streptocyclin, Ciprofloxacinwas reported by [30] to control contamination during *in vitro* propagation of different bamboo species like *B. tulda, B. wamin*and *B. balcooa*. There was only one report [64] who used Kanamycin and streptomycin sulfate as media component during multiplication of *Guadua angustifolia* Kunth. All the earlier works, researchers used broad spectrum antibiotics for decontamination. Reports on application of antibiotic after identifying the bacteria,are available in case of *Ilex dumosa* [55] , *Aglaonema* [27], *Guadua angustifolia* Kunth [64]. No attempts have been taken to use antibiotic after identifying bacteria in bamboo (except in *Guadua angustifolia* Kunth). In the era of genomics, bacteria and fungus may easily be identified by using the molecular techniques. It may be suggested to use antibiotics during micropropagation after proper identification of the endophytes to get substantial results. The conserved sequence in 16S rDNA for bacteria and Internal transcribed spacer (ITS) sequence for Fungus may be utilized for identification and there by antibiotic may be selected for its eradication.

CONCLUSION

The endophytic contaminants are serious concern in micropropagation. Presence of contaminants in culture affects the growth of plant *in vitro*. Plant tissue culture is an expensive method, so contamination frees cultures are need to be developed to get desirable profit. There several antibiotics were already reported by several authors to eliminate the endophytic contaminants during tissue culture of many plants due to failure of general sterilization method. Antibiotic as a media component is more effective as evident from the report of earlier workers on different plants. But addition of antibiotic within media may lead to phytotoxic effect on plant. So, broad spectrum antibiotic with low phytotoxicity is only desirable to eliminate the endophytic contaminants. For this reason there need to find out the minimum inhibitory concentration of antibiotic against particular microorganisms after its proper identification using the most reliable molecular technique like 16S rDNA. There need further research work particularly on bamboo since each antibiotic has different mode of action, solubility etc.

Table 1: Antibiotics used in bamboo tissue culture

Bamboo species	Antibiotic	Concentration	Duration (Minutes)	Reported by
B.balcooa	Gentamycine	0.1%	Depending upon the collection time .	[84]
	Bacteriomycine	0.5%	15 minutes	[89]
B. nutans	Streptomycin Sulfate	0.05%	20-25 minutes	[68]
	Streptomycin Sulphate	0.1%	5 minutes	[85]
Dendrocalamus giganteus	Streptomycin and Kanamycin	Streptomycin0.01 % (w/v), Kanamycin 0.05% (w/v)	30 minutes	[92]
*D.*membraneous	Streptomycin Sulfate	0.25%	45 minutes	[91]
Guadua angustifolia	1.Agri-mycin (Surface sterilants). 2. PPM (Media component)	2 gm/l 2 ml./l	10 minutes	[67]
Guadua angustifolia Kunth	Kanamycin and streptomycin sulphate	10 μg/ml each	Act as media component	[64]

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